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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The effects of long-term treatment with indole-3-carbinol (I3C) and/or tamoxifen (TAM) on caspase activities in mammary glands and tumors were examined. Both controls and DMBA-pretreated rats were treated 3 doses per week, up to 52 doses, with (1) Vehicle, (2) TAM (10 µg per rat), (3) I3C (250 mg/kg) and (4) TAM+I3C, respectively. Rats were sacrificed at selected intervals for mammary glands and tumors. Colorimetric caspase assay shows that in normal mammary glands, I3C increased caspase activities earlier than TAM, and TAM+I3C treatment induced additive levels of caspase activity only at an early treatment phase. At the late treatment stage, TAM reached its greatest induction of caspase activities, and induced caspase activities 3-4-fold greater than TAM+I3C. I3C induced significantly greater caspase activities in mammary glands of tumor-free DMBA-treated rats than of tumor-bearing rats. None of the treatments significantly induced caspase activities in mammary tumors. 3,3'-diindolylmethane (DIM) failed to induce greater caspase activities in a short-term treatment than vehicle. The data suggest that I3C might be prophylactic before mammary tumors develop but it is not a promising adjuvant agent with TAM in induction of caspase activities in mammary tumors. DIM may not be the active form of I3C in the induction of apoptotic activities by a short-term treatment.				
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Introduction

Indole-3-carbinol (I3C), a natural phytochemical from cruciferous vegetables, and its gastric acid condensation products, such as 3,3'-diindolylmethane (DIM) and 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (LTr-1), have been shown effective in suppression of breast cancer *in vivo* (rodents) or *in vitro* (cell cultures). Several mechanisms of action of these agents have been investigated, including: a) modulations of phase I and phase II enzymes by I3C/DIM leading to detoxification of carcinogens (Bradlow *et al.*, 1991, Wortelboer *et al.*, 1992); b) inhibition of cyclin-dependent kinase (CDK) 6 expression by I3C and induction of G1 cell cycle arrest (Cover *et al.*, 1998); c) promotion by I3C of Bax translocation to mitochondria to induce apoptosis (Sarkar *et al.*, 2003); and d) inhibition by LTr-1 of the growth of both ER(-) and (+) breast cancer cells (Chang *et al.*, 1999). It is thus suggested that the tumor inhibitory effects of I3C or its products may be ascribed to signal transduction pathways independent of ER status of the tumor, and thus, may be different from the mechanism of action elicited by tamoxifen (TAM). TAM blocks estrogen receptor (ER) and therefore has selective efficacy in patients with ER(+) mammary tumors. TAM was also reported to induce caspase activities preceding apoptosis in rat mammary tumors *in vivo* as well as in both ER(-) and ER(+) human breast cancer cells *in vitro* (Mandlekar *et al.*, 2000).

Apoptosis is mediated through activation of either caspase 8 (C8) for receptor-mediated cell death pathway (extrinsic) or caspase 9 (C9) for mitochondrial cell death pathway (intrinsic). The downstream effectors, such as caspase 3 (C3), caspase 6 (C6) and caspase 7 (C7), are subsequently activated by upstream initiators C8 and/or C9 and then participate in a cleavage cascade of a variety of cellular proteins, resulting in systematic disassembly of dying cells. Previous studies from this laboratory showed that caspase activities in the mammary glands were increased by short-term treatment of rats with I3C, but were unaffected by long-term treatment with I3C in mammary adenocarcinomas (Zhang and Malejka-Giganti, 2003).

Many anticancer chemicals are believed to trigger apoptosis through diverse signal pathways. Thus, treatment with two or more drugs that have different but complementary mechanisms of action may be advantageous to exert maximum suppression effects on tumorigenesis. In this study, we investigated whether a long-term treatment of rats with I3C induces apoptosis in the mammary glands, and whether an adjuvant therapy with TAM further modifies effects of I3C in mammary glands and tumors. Hence, caspase activities were examined in mammary glands and tumors of TAM-, I3C- and TAM+I3C-treated rats. Since the major I3C product *in vivo* is acid-condensed dimer DIM, the effects of short-term treatment with DIM on caspase activities in mammary glands were also examined.

Study design

Two weeks after one oral dose of 65 mg/kg bw of 7,12-dimethylbenz[α]anthracene (DMBA) was given to 7-wk-old Sprague-Dawley female rats for initiation of mammary carcinogenesis, rats were divided into four groups and treated 3 times per week, up to 52 times, with: (1) Vehicle I of TAM (Veh I, 10% ethanol in olive oil at 0.1 ml/rat, s.c.) + Vehicle II of I3C (Veh II, 20% ethanol in olive oil at 2.5 ml/kg bw, i.g.) [VehI+VehII], (2) TAM (10 μ g per rat in 50 μ l Veh I, s.c.) + Veh II [TAM+VehII], (3) Veh I + I3C (250 mg/kg in Veh II, i.g.)

[VehI+I3C] and (4) TAM + I3C as above [TAM+I3C] (*Table 1*). Control (DMBA-untreated) rats also consisted of four groups treated as above. At intervals of about three weeks, two to four rats from each of the control groups were sacrificed. The rats in DMBA-treated groups were palpated for breast tumors every week. When the tumors reached about 1cm in diameter, the rats were sacrificed. The non-tumor bearing rats from each treatment group were also sacrificed at the corresponding time interval. The mammary glands and tumors were isolated for histological evaluation and biochemical studies. In DIM study, 7-week-old female rats were treated by oral gavage with DIM (42 mg/kg bw) in Veh II or only Veh II once daily for 4 days before sacrifice.

The snap frozen mammary glands and tumors were homogenized and extracted for protein. After the protein concentration was adjusted at ~3mg/ml, caspase activities of C3+7, C6, C8 and C9 were measured using modified colorimetric caspase assay system (Promega, USA). The mean natural logarithms of activity \pm standard deviation were statistically analyzed using a one-way analysis of variance (SPSS, USA) to compare the effects of treatments and treatment durations. The significance level was set at $P=0.05$.

Results

Tissue differences (*Table 2*)

Caspase activities were significantly greater in mammary tumors (DMTs) than in mammary glands (CMG and DMG groups) (7.0-, 8.7-, 7.3- and 10.3-fold greater for C3+7, C6, C8 and C9, respectively, $P<0.00001$), irrespective of the treatment and treatment duration. In CMGs, C3+7 and C8 activities were significantly greater (2.0-fold, $P<0.0001$ and 1.9-fold, $P=0.0003$, respectively) than in DMGs. The activities of initiator caspases in DMG_(t+) were significantly different from those in DMG_(t-) (C8: 1.8 times lower, $P<0.00001$; C9: 2.5 times greater, $P<0.0001$).

Effects of treatment

At the early treatment phase (17 doses during ~38 days) in CMG, caspase activities in TAM+I3C group 4 were about the sum of those in TAM group 2 and I3C group 3, and activities of C3+7 and C6 were significantly higher (4.4-fold, $P=0.088$ and 2.7-fold, $P=0.039$, respectively) in group 4 than in group 2.

At the very late treatment phase (52 doses during ~118 days), caspase activity levels in CMG were the highest in TAM group 2 and lowest in TAM+I3C group 4 (C3+7: 3.9-fold, $P=0.064$; C6: 2.9-fold; C8: 5.1-fold, $P=0.231$; C9: 4.1-fold, $P=0.085$) and were similar between I3C group 3 and Vehicle group 1.

I3C treatment (group 3) induced significantly greater C6 and C9 activities (1.5-fold, $P=0.045$ and 2.3-fold, $P=0.019$, respectively) in DMG_(t-) than in CMG. Group 3 had greater activities of C3+7, C6 and C9 (3.2-fold, $P=0.0003$; 1.7-fold, $P=0.04$; 2.9-fold, $P=0.0008$, respectively) in CMG compared to DMG_(t+). I3C treatment significantly increased C3+7, C6 and C9 activities (2.9-fold, $P=0.0018$; 2.6-fold, $P=0.0002$ and 6.8-fold, $P<0.0001$, respectively) in DMG_(t-) compared to DMG_(t+) (*Table 2*), especially at the late phase of treatment. Vehicle and TAM

groups had significantly lower C3+7 and C8 activities in DMG_(t-) than in DMG_(t+), especially at early phases.

I3C treatment increased caspase activities (C3+7 and C6) in DMGs significantly greater than other treatments at the late treatment phase. Compared to other treatments, I3C increased the caspase activities in DMG_(t-) as follows for the overall treatment duration: 2.5- to 3.3-fold greater than TAM for all the caspases, $P \leq 0.002$; 1.6- and 1.8-fold greater than TAM+I3C for C6 and C8, $P \leq 0.05$; 3.3- and 3.4-fold greater than TAM+I3C for C3+7 and C9, $P = 0.07$ and $P = 0.08$, respectively (Table 2).

Effects of treatment phase

In CMGs, caspase activities of TAM-treated rats increased with treatment duration (significant for C3+7 and C6) and were approaching summit ≥ 118 days, but peaked in both I3C and TAM+I3C groups at about 38 days and then decreased (C8 for I3C group, all caspases for TAM+I3C group).

In DMGs, the levels of caspase activities were also associated with treatment phase. In treatment group 1, the C8 caspase activities in DMGs increased with treatment duration. During the mid-term treatment phases, I3C showed lower induction effects on C6 and C9 than during both early and late phases.

In DMTs, I3C increased caspase activities at an earlier phase of treatment than TAM did. At the late treatment phase, caspase activities in TAM+I3C treatment group were lower than at the early phases and became the lowest among the four treatment groups. No significant association was found between caspase activities and tumor latent period, tumor mass, or pathology.

Effects of short-term treatment with DIM

Caspase activities of C3+7, C6 and C9 were insignificantly lower in DIM-treated group comparing to control group, but the decrease of C8 activity in the DIM-treated group was significantly (2.0-fold, $P = 0.07$) lower.

Key Research Accomplishments

- Treatment with I3C was found to increase caspase activities in normal mammary glands only at an early treatment phase (up to 17 doses during ~38 days); TAM induced caspase activities later than I3C and reached the greatest induction at the late treatment phase (52 doses during ~118 days).
- Combination treatment with I3C and TAM appears to have an additive effect on the induction of caspase activities in normal mammary glands only at the early treatment phase.
- Long-term treatment with I3C (up to 52 doses during 118 days) was found to increase caspase activities in mammary glands of tumor-free DMBA-treated rats.
- Caspase activities were found to be the highest in mammary tumors. The caspase activities were higher in mammary glands of normal rats than in those of DMBA-treated rats.

- In contrast to a short-term treatment with I3C, DIM had no induction effect on caspase activities in normal mammary glands, confirming the earlier results of Zhang and Malejka-Giganti (Anticancer Res. 2003, 23: 2473-9)

Conclusions

Treatments with TAM, I3C or TAM+I3C resulted in different levels of induction of caspase activities in mammary glands and tumors, and the magnitude of the effects was associated with treatment phase, DMBA initiation and tumor development status.

In the mammary glands of normal female Sprague-Dawley rats, I3C increased caspase activities to a greater extent and at an earlier phase than TAM did. The combination treatment of TAM+I3C revealed additive induction of caspase activities at an early treatment phase, but at a later phase these two compounds may be inhibitory to each other in the induction of caspase activities.

Generally, I3C increased caspase activities to a greater extent than other treatments in the mammary glands from tumor-free DMBA-treated rats, especially at late treatment phases. The greater increases in caspase activities induced by I3C in the mammary glands from tumor-free rats than tumor-bearing rats suggest that I3C might be sustaining a tumor-free status of the mammary gland in DMBA-treated rats.

TAM and/or I3C failed to significantly increase caspase activities in the mammary tumors (DMTs). On the contrary, these two compounds in combination appeared to decrease caspase activities in DMTs at the late treatment phase.

Considering an increase in apoptosis, the protection virtue of I3C *in vivo* might be prophylactic in that I3C protects the mammary glands from the effects of DMBA in tumor-free rats, or prevents or postpones the DMBA-induced carcinogenesis. I3C does not appear to be a promising adjuvant agent with TAM in induction of caspase activities in mammary tumors. The failure of DIM in inducing caspase activities in mammary glands suggests that the induction of apoptotic activities by short-term treatment with I3C cannot be attributed to DIM.

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Appendices

Table 1: The treatment groups and corresponding tissue designation: CMG, mammary glands from control groups; DMG, mammary glands from DMBA-treated groups; DMG_(t-), mammary glands from tumor-free rats of DMBA-treated groups; DMG_(t+), mammary glands from tumor-bearing rats of DMBA-treated groups; DMT, DMBA-induced mammary tumors.

Treatment group	Control	DMBA-treated			
	Mammary Glands	Mammary Glands			Mammary Tumors
		All	Non-tumor bearing	Tumor bearing	
1: Veh I+Veh II	CMG	DMG	DMG _(t-)	DMG _(t+)	DMT
2: TAM+Veh II	CMG	DMG	DMG _(t-)	DMG _(t+)	DMT
3: Veh I+I3C	CMG	DMG	DMG _(t-)	DMG _(t+)	DMT
4: TAM+I3C	CMG	DMG	DMG _(t-)	DMG _(t+)	DMT

Table 2: Caspase activities in mammary glands and tumors

Caspase	Treatment group ^a	Caspase activities (Mean±SD) ^b in different tissue types				
		CMG	DMG	DMG _(t-)	DMG _(t+)	DMT ^c
Caspase 3+7	1	340±265	128±85	92±73	151±87	1878±907
	2	380±417	160±134	126±119	221±144	1800±981
	3	466±417	283±339	423±439 ^d	144±75	1985±895
	4	368±293	217±141	228±171	203±97	2005±1155
Caspase 6	1	85±46	69±32	64±35	72±32	825±427
	2	99±81	77±42	68±41	93±42	791±440
	3	110±72 ^e	118±89	170±100 ^d	67±27	794±403
	4	96±50	99±46	105±55	91±34	854±480
Caspase 8	1	69±63	39±25	23±19	49±23	399±217
	2	78±72	33±31	17±13	62±33	389±246
	3	93±82	50±35	52±45 ^d	48±22	408±202
	4	74±54	40±28	29±28	55±20	453±269
Caspase 9	1	41±28	24±18	27±21	22±16	494±234
	2	53±66	38±35	40±38	33±32	467±250
	3	57±45 ^e	76±115	132±143 ^d	19±16	498±222
	4	45±38	53±35	66±37	37±25	529±287

^a Treatment groups are listed in Table 1.

^b The quantity of p-nitroaniline in nmol liberated per mg protein per 24 hr. The values are the means from the assays of all time points during the treatment period.

^c All values in DMT column were significantly greater than the corresponding values in CMG, DMG, DMG_(t-), DMG_(t+) columns.

^d Denotes significant greater increases of caspase activities in DMG_(t-) by Veh+I3C treatment (group 3) than by other treatment groups.

^e Denotes significant differences between CMG and the respective DMG_(t-) and DMG_(t+) values.